Stereoselective Biocatalysis. A mature technology for the asymmetric synthesis of pharmaceutical building blocks

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Dedicated to Professor Vicente Gotor on occasion of his 70th birthday

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Abstract

Biocatalysis is gaining increasing attention in the academic and industrial sector due to the possibility of developing highly stereoselective transformations in a sustainable manner. The creation of stereogenic centers in organic synthesis is not trivial and multiple approaches have been disclosed based on
organometallic and organocatalytic methods with the use of day by day more complex catalysts to induce asymmetry in selected transformations. The intrinsic chirality of enzymes makes them powerful tools for the development of stereoselective transformations, catalysing a wide range of chemical reactions due to the high abundance and diversity of enzymes in nature. In addition, the enormous advances in rational design and molecular biology methods have opened up the possibility to create more robust and versatile biocatalysts, which have improved the initial activities displayed by wild-type enzymes. Therefore, their applicability has been widely increased in terms of reaction conditions, substrate specificity, activity and selectivity among others. All these properties have attracted the industrial sector, which has taken advantage of the enzyme selectivities in multiple scenarios. Herein, the focus has been put in recent developments of stereoselective transformations for the synthesis of valuable building blocks towards the production of pharmaceuticals and biologically active natural products.

1. Introduction

Demands for enantiopure compounds in the pharmaceutical industry continue rising because of the restrictions applied by national regulation agencies. The synthesis of single enantiomers of intermediates and drugs is of special interest, not only because of side-effects caused by drug enantiomers but also due to the low effectivity when administrating racemates. In this context, Biocatalysis provides efficient and selective transformations for the development of sustainable synthetic methods towards a broad family of chiral compounds (Drauz 2012). Further, the generation of wastes is dramatically decreased in many cases when compared with conventional methods due to the high atom efficiency under mild operational conditions displayed by biocatalytic methods (Ni 2014).

The aim of this review is covering recent stereoselective biotransformations for the production of valuable building blocks in the synthesis of pharmaceuticals and biologically active products. Thus, we have put our focus in the last three years (2015-2017), trying to present an actual scenario about the role
that enzymes are playing in the pharma sector. To achieve this, a set of examples has been covered and classified based on the type of biocatalyst. Special attention has been paid to the use of hydrolases due to their ability to catalyse classical and dynamic kinetic resolutions of racemates, but also desymmetrisation of meso and prochiral compounds. In a first section, the use of lipases, esterases, proteases, lactamases, epoxide hydrolases and amidases will be discussed as they give practical solutions for the asymmetrisation of a variety or organic compounds. Next, the broad use of alcohol dehydrogenases for the stereoselective reduction of carbonyl compounds will be described, to later disseminate the potential of other enzyme classes such as different oxidoreductases, lyases and transferases. Then, the performance of concurrent one-pot processes using various enzymes will be explained, which is a currently a demanding trend in synthesis design.

Interestingly, in many cases there is more than one strategy to reach a synthetic target, so the development of complementary biotransformations will be finally analysed in order to compare recently described synthetic methodologies and find the best solution for the synthesis of a certain drug. The proper design of retrosynthetic analysis is of crucial importance to establish efficient and sustainable chemoenzymatic routes.

2. Hydrolases.

The possibility to carry out not only hydrolytic procedures but also synthetic reactions in organic solvents is of paramount importance for the design of stereoselective synthetic routes. In this context, lipases are the hydrolytic enzymes that have attracted much attention by means of acetylation, esterification, transesterification, aminolysis and ammonolysis reactions (Méndez-Sánchez 2016). In this section, we firstly describe the main achievements using the most recurrent strategy, the kinetic resolution of alcohols through acylation using activated esters to later disclose the application of hydrolytic procedures. Finally, dynamic kinetic resolutions or desymmetrisation reactions will be also revised.
2.1. Hydrolases in classical and dynamic kinetic resolutions.

Classical kinetic resolutions (KRs) of racemates are limited to a theoretically maximum 50% yield of an enantiopure product but, at the same time, they provide access to both enantiomers, which is extremely important for biological evaluation. For many years, biocatalytic kinetic resolutions using hydrolases have been playing a pivotal role in the synthesis of bioactive natural products and derivatives. For instance, the (+)-Artabotriol sugar is a fundamental building block for the synthesis of diverse enantiomerically pure natural products. The precursor (±)-2-hydroxy-3-methylenesuccinate has been resolved through an acetylation reaction using the *Pseudomonas cepacia* lipase commercialised by Amano (Amano PS) and 5 equivalents of vinyl acetate (VinOAc) as acyl donor in acetone (Scheme 1). After 3 days at 25 ºC, the reaction with 2 g of substrate led to the (+)-acetate (46% yield, 98% ee) and the (−)-alcohol (54% yield, 94% ee) that were separated by column chromatography (Batwal 2016).

![Scheme 1. Lipase-catalysed KR of racemic dimethyl-3-methylenesuccinate for the synthesis of (+)-Artabotriol and other derived products.](image-url)

The (+)-Artabotriol has been obtained in three steps from the optically active acetate with 31% overall yield and, in addition, other bioactive natural products have been synthesised: (+)-Grandiamide D (12%...
yield in 4 steps), (−)-Tulipalin B (23% yield in 3 steps), (+)-Spiranthudiol (13% yield in 3 steps) and (+)-Arabotrioalcaffeate (14% yield in 3 steps).

The same enzyme (Amano PS) has been successfully applied in terpenoid-derived natural products synthesis through the resolution of racemic Aristegone B (Scheme 2). Again, 5 equivalents of VinOAc were used, developing the acetylation in acetone and, after 36 h at 25 ºC, the (+)-acylaristelegone B was obtained in 46% yield and 96% ee and the (−)-Aristegone B in 54% yield and 94% ee (Batwal 2015).

![Scheme 2. Lipase-catalysed acetylation of racemic Aristegone B.](image)

γ-Cyclogeranyl unit is a common feature in natural products, including (+)-trixagol and (+)-luffarin-P, which are precursors of antibacterial terpenes (Scheme 3). The lipase-catalysed KR of γ-cyclogeraniol has been studied using a variety of lipases and vinyl esters, finding in general low selectivites, which is not surprising due to the difficulty when the stereogenic center is far from the reactive group (Blasco 2014 and Cunha 2015). The use of CAL-B and vinyl propionate was a good combination, but because of the inactivation of the CAL-B at prolonged times, the reaction was left for 7 days at room temperature and, after this time, the mixture was filtrated and the solvent evaporated. Then, fresh CAL-B, vinyl propionate and solvent were added and the reaction was stirred for one more week. Finally, the (R)-propionate was obtained with 32% ee but, satisfyingly, the remaining alcohol was recovered in enantiopure form in 23% yield (Fujii 2016). More recently, lipases such as the one from Candida rugosa lipase (CRL), AK from Pseudomonas fluorescens, porcine pancreas lipase (PPL), CAL-B or PSL were tested in the resolution of the related racemic α-cyclogeraniol using green solvents such as 2-methyltetrahydrofuran and cyclopentyl methyl ether (Belafriekh 2017). In all cases, poor to moderate
selectivities were also found \((E= 1-19)\).

![Scheme 3](image)

**Scheme 3.** Lipase-catalysed acetylation of \(\gamma\)-cyclogeraniol.

Another natural product is Amphirionin-4 with remarkable proliferation-promoting activity in marine bone marrow stromal ST-2 cells (Scheme 4). Racemic \(cis\)-3-hydroxy-5-methyldihydrofuran-2\((3H)\)-one has been identified as a good candidate for giving access to two intermediates for the synthesis of both Amphirionin-4 enantiomers. Thus, after resolution with vinyl acetate and the PS-30 lipase, the \((-)\)-acetate was obtained in 50% yield and 92\% ee, while the remaining \((+)\)-hydroxylactone was recovered in 47\% yield and 94\% ee (Ghosh 2017).

![Scheme 4](image)

**Scheme 4.** Lipase-catalysed resolution of racemic \(cis\)-3-hydroxy-5-methyldihydrofuran-2\((3H)\)-one.
Atenolol is a cardioselective β-blocker used in the treatment of high blood pressure, angina and myocardial infections (Scheme 5). Its stereoselective synthesis is of crucial importance because the major activity resides in the (S)-enantiomer, while the (R)-enantiomer has potential adverse effects. *Candida antarctica* lipase B (CAL-B) has displayed excellent levels of activity in the resolution of two Atenolol precursors, namely 2-[4-(3-chloro-2-hydroxypropoxy)phenyl]acetamide ($E = 220$) and 2-[4-(3-bromo-2-hydroxypropoxy)phenyl]acetamide ($E = 278$). In both cases the (S)-ester and the (R)-alcohol were isolated in >93% ee (Lund 2016). Interestingly, the solubility of the chlorinated precursor in organic solvents has been identified as a key issue in its enzymatic reactivity, so ionic liquids (ILs) have been used as cosolvents in the acetylation reaction (Dwivedee 2015). After looking at different parameters (source of lipase, solvent, reaction time, acyl donor, temperature, enzyme loading and substrate concentration), *Candida antarctica* lipase A (CAL-A) immobilised as crosslinking enzyme aggregates has been found as the most effective biocatalyst in combination with vinyl acetate. Using a mixture of toluene and [emim][BF$_4$] (90:10, v/v), a conversion close to 50% was reached, yielding product and substrate with excellent selectivity.

![Scheme 5. CAL-B catalysed resolution of Atenolol precursors using vinyl butanoate as acyl donor.](image)

Alternatively, the lipase-catalysed resolution of the own racemic Atenolol has been described by other
authors. On the one hand, *Pseudomonas fluorescens* lipase (PFL) was investigated as enzyme for the acylation reaction using vinyl acetate (Agustian 2016). An exhaustive study of the reaction conditions was conducted including parameters such as agitation speed, substrate concentration, temperature, ratio of vinyl acetate and enzyme loading. On the other hand, *Candida rugosa* lipase (CRL) has been immobilised onto two different chitosan magnetic nanoparticles, obtaining a good enantioselectivity ($E=67$) in the resolution of Atenolol using isopropenyl acetate and toluene (Sikora 2016). Interestingly, the reused immobilised lipase maintained the stability after five reaction cycles.

Eslicarbazepine, also known as (S)-Licarbazepine, is the pharmacologically active form of antiepileptic drugs such as carbamazepine and oxcarbazepine (Scheme 6). The resolution of its racemate has been studied using 10 lipases, finding CRL as the most efficient one (El-Behairy 2016). The best conditions were found using 2 equivalents of vinyl benzoate as acyl donor and TBME as solvent and, after 5 days at 40 °C, the (R)-ester and the (S)-alcohol were isolated in 77 and 97% ee, respectively. Unfortunately, the complementary resolution of Licarbazepine esters by a hydrolytic procedure led in all cases to very poor selectivities ($E<5$).

![Scheme 6. Stereoselective acylation reaction of Licarbazepine with CRL and vinyl benzoate.](image)

Dynamic kinetic resolutions (DKRs) are valuable strategies for the synthesis of enantiopure compounds because of the possibility to obtain a single enantiomer in theoretically 100% yield when starting from a racemate. In this context, the chemoenzymatic synthesis of the antitussive drug L-Cloperastine has been described, identifying the DKR of phenyl[4-(trimethylsilyl)phenyl]methanol as the key step for the
introduction of chirality (Scheme 7). Therefore, the DKR of 24 diarylmethanols has been successfully achieved using the lipoprotein lipase from *Burkholderia species* coated with a dextrin and an ionic surfactant (LPL-D1) in combination with isopropenyl acetate and a ruthenium complex. The diarylmethyl acetates thus obtained were isolated in high yields (71-96%) with enantiopurities of 90-99% ee. Particularly, the \((R)\)-phenyl\[4-(trimethylsilyl)phenyl\]methyl acetate was isolated in 82% yield and 96% ee after 72 h at 40 ºC (Lee 2015).

![Scheme 7](image)

**Scheme 7.** DKR of phenyl[4-(trimethylsilyl)phenyl]methanol for the chemoenzymatic synthesis of L-Cloperastine.

The use of organic solvents and neoteric solvents has been extensively described in this section for the kinetic resolution of chiral drug intermediates by transesterification reactions. Now, the use of hydrolytic procedures will be undertaken, which can be carried out in both aqueous medium or alternatively in organic solvents using water as nucleophile.

Brivaracetam is an antiepileptic drug, its use recently approved in Europe and USA as adjunctive therapy in the treatment of partial onset seizures in patients with epilepsy (Scheme 8). The enzymatic and salt resolutions of adequate intermediates have been recently studied, paying special attention to the hydrolase-catalysed resolution in hydrolytic conditions (Schüle 2016). After a screening of 30 lipases, 30 esterases, 15 proteases and one acylase, the most promising results were found with the *Bacillus subtilis* protease in a phosphate buffer at pH 7.5, yielding the remaining \((S)\)-ester in 99% ee and the \((R)\)-succinic acid derivative in 95% ee \((E= 210)\). A deep study of the reaction conditions was then performed, searching for suitable scale-up conditions including the work-up and isolation of the chiral
products. Best conditions for the 1-kg substrate biotransformation were found using water as solvent (4.5 L/kg), yielding 394.4 g of the (R)-2-propylsuccinic acid 4-tert-butyl ester in 42% isolated yield with 97% ee.

**Scheme 8.** Hydrolase-catalysed KR of a Brivaracetam precursor by hydrolysis.

Moxifloxacin is a fluoroquinolone antibacterial agent, specially used in respiratory infections such as pneumonia, chronic sinusitis and bronchitis (Scheme 9). A key Moxifloxacin precursor has been prepared by hydrolytic resolution of racemic *cis*-dimethyl-1-acetylpyrrolidine-2,3-dicarboxylate at 80-g scale using soluble CAL-B (Ramesh 2015). Thus, after an extraction protocol the remaining enantiopure (−)-diester was obtained in 46% yield. Due to a migration reaction of the methoxy group between the C-2 and C-3 positions, the resulting monoester was recovered as a mixture of monomethyl esters in 52% yield. These were esterified with thionyl chloride and methanol to measure the optical activity as the corresponding diester (85% ee). This protocol represents a clear advantage compared with the use of the immobilised CAL-B as only 16 h instead of 140 h were required for the completion of the reaction. It must be also mentioned that only the *N*-acetyl dimethyl ester was a good substrate for CAL-B as either the methyl diester without *N*-acetylation or the ethyl, *n*-propyl or *n*-butyl diesters were recognised by the enzyme.
Amino acids and their derivatives, including β- and γ-lactams, are a highly important class of organic compounds due to their multiple applications in medicinal chemistry. A key reaction is the ring-opening of lactams which is naturally catalysed by lactamases, although lipases have also been applied in this type of reactions. Abacavir and carbovir belongs to the carbocyclic nucleoside family, which possess antibiotic and antiviral activities (Scheme 10). A common precursor for both is the 2-azabicyclo[2.2.1]hept-5-en-3-one also known as Vince lactam, and its KR has been possible by means of a hydrolytic procedure using a (+)-γ-lactamase from *Bradyrhizobium japonicum* USDA 6 (Gao 2015). This enzyme was cloned, purified and characterised, to later hydrolyse the γ-lactam, yielding the desired enantiopure lactam and the amino acid in 50% conversion. An exhaustive search was performed towards the optimum reaction temperature, pH, presence of ions and substrate concentration. Similarly, a non-heme chloroperoxidase from *Streptomyces viridochromogenes* DSM 40736 with promiscuous (−)-γ-lactamase activity (SvGL) has been successfully applied for this transformation but with complementary stereochemistry (Yin 2016). The reaction was carried out using 4.4 g of the racemic lactam (4 M) in phosphate buffer at pH 7.0, leading to a 49.8% conversion within 11 h, and isolating 2.1 g of the (+)-lactam (48% yield and >99% ee) that is a potential precursor of Melogliptin a DP-IV
inhibitor tested for treatment of type II diabetes. Satisfyingly a space time yield (STY) of 458 g L⁻¹ d⁻¹ was reached for a remarkable E factor value of 5.7 (2.9 excluding water).

![Chemical structures of Abacavir and Melogliptin](attachment:image)

**Scheme 10.** Hydrolysis of 2-aza-bicyclo[2.2.1]hept-5-en-3-one using a (+)-γ-lactamase from *Bradyrhizobium japonicum* USDA 6 (left) or a (−)-γ-lactamase from *Streptomyces viridochromogenes* (right).

CAL-B has been also studied in the ring-opening of lactams, finding excellent selectivities in the hydrolysis of some of them including the Vince lactam previously cited. Interestingly, the use of the $N$-hydroxymethyl group allows the activation of the lactam, leading to the production of the desired products in high optical purities and shorter reaction times in comparison with the unsubstituted lactam (Galla 2016a). As the ring-opened amino acid is formed, the $N$-hydroxymethyl group undergoes spontaneous degradation in the presence of benzylamine in the reaction medium, leading to the formation of both lactam and free amino acid with very high enantiomeric excesses (Scheme 11).
Scheme 11. CAL-B catalysed hydrolytic resolution of γ-lactams protected with N-hydroxymethyl group with spontaneous deprotection of the amino acid product.

The use of the N-hydroxymethyl group and identical reaction conditions have been applied by the same authors to the resolution of additional four β-lactams, which have potential use as building blocks in the synthesis of different drugs such as the novel antitumoral CEP-28122 (Forró 2016, Scheme 12).
Scheme 12. CAL-B catalysed hydrolytic resolution of β-lactams precursors of pharmacologically active compounds protected with the N-hydroxymethyl group.

The resolution of 3,4-disubstituted β-lactams with free N-H has been also studied using CAL-B as biocatalyst, and in this case a large excess of water as nucleophile is required (25 equivalents, Galla 2016b). After a screening of water amount, solvent and temperature, the best conditions were found at 70 °C observing a dramatic influence in the reactivity and selectivity depending on the substituents (Table 1). Remarkably, the enantiopure (2R,3S)-amino acid with $R^1 = $ Bn and $R^2 = $ H is a key intermediate in the synthesis of the paclitaxel (sold as the brand name Taxol) side-chain, which is a chemotherapy medication used in the treatment of several types of cancer.
Table 1. Preparative-scale resolution of 3,4-disubstituted β-lactams.

\[
\begin{array}{|c|c|c|c|c|c|c|}
\hline
R^1 & R^2 & c (%) & E & Yield lactam (%) & ee lactam (%) & Yield amino acid (%) & ee amino acid (%) \\
\hline
Bn & 4-Cl & 50 & >200 & 35 & 98 & 30 & 99 \\
Bn & H & 50 & >200 & 48 & 98 & 47 & 99 \\
Ph & 4-Cl & 66 & 12 & 16 & 98 & 61 & 50 \\
\hline
\end{array}
\]

Racemic 4-phenylazetidin-2-ones can be also resolved by using methanol instead of water as nucleophile in dry organic solvents (Table 2). This alcoholsysis reaction leads to the remaining lactams and the corresponding methyl esters in conversions close to 50% and excellent selectivities when the nitrogen atom was unprotected or protected with the acetyl or chloroacetyl group (Sundell 2015). A great influence has been observed depending on the N-substitution, in fact poor or none conversions were found with the N-Boc, N-allyl, N-methoxybenzyl and N-tert-butylidiphenylsilyl derivatives due to either steric effects or poor N-activation.

Table 2. CAL-B catalysed resolution of racemic 4-phenylazetidin-2-ones.

\[
\begin{array}{|c|c|c|c|c|c|}
\hline
R & time (h) & c (%) & Yield lactam (%) & ee lactam (%) & Yield amino ester (%) & ee amino ester (%) \\
\hline
H & 96 & 47 & 38 & 94 & 36 & >99 \\
Ac & 24 & 50 & 40 & >99 & 41 & >99 \\
ClCH_2CO & 24 & 46 & 26 & 91 & 25 & >99 \\
\hline
\end{array}
\]

Propranolol is a widely known β-blocker, whose (S)-enantiomer is 100-times more activity than its counterpart in blocking β-adrenergic receptors. Xu and co-workers have recently reported the use of an
engineered epoxide hydrolase (EH) from Bacillus megaterium for the hydrolytic resolution of α-naphthyl glycidyl ether in a preparative scale (20 g of substrate in a 100 g/L concentration). Using a biphasic system composed by \( ^i\)Pr\( _2\)O, isoctane and a buffer with a surfactant (Tween-80), the (S)-epoxide (45% yield, STY of 136 g L\(^{-1}\) d\(^{-1}\)) and the (R)-diol (42% yield, STY of 139 g L\(^{-1}\) d\(^{-1}\)) were isolated in enantiopure form for a 70000 total turnover number (TTN) of the enzyme, which is much higher than the ones described with other biocatalysts. Both optically active compounds served for the immediate synthesis of Propranolol enantiomers (Kong 2015, Scheme 13). In another example of epoxide opening, site-saturation and site-directed mutagenesis were performed over the EH from Agromyces mediolanus ZJB120203, finding a triple mutant (W182F, S207V and N240D) with improved activity against epichlorhydrin (E value changed from 12.9 with the wild-type to 90.0 for this mutant). In this manner, the (S)-epoxide was isolated in enantiomerically pure form and 40.5% yield after 90 minutes of reaction at 450 mM substrate concentration (Xue 2015).

![Scheme 13](image)

**Scheme 13.** Epoxide opening with a EH for the chemoenzymatic synthesis of Propranolol enantiomers.

### 2.2. Hydrolases in desymmetrisation reactions.

Besides resolutions, hydrolases are able to carry out desymmetrisation reactions. As occurs with DKRs, this is an interesting strategy as the desired enantiopure product can be obtained in 100% theoretically yield (García-Urdiales 2011). The main difference resides in the starting material: a racemate is transformed in DKRs while a meso or a prochiral compound is employed in desymmetrisation reactions. (-)-Alloyohimbane and (-)-Yohimbane are two interesting alkaloids that display a wide range of
pharmacological activities, such as antihypertensive and antipsychotic (Ghosh 2016). Their enantioselective synthesis have been performed using a common building block, [(1R,6S)-6-(hydroxymethyl)cyclohex-3-en-1-yl]methyl acetate, which was obtained by enzymatic hydrolysis of the meso-diacetate precursor (Scheme 14). PPL catalysed the hydrolytic reaction using 51 g of substrate in a phosphate buffer pH 7 at 23 ºC, obtaining the desired monoacetate in 84% yield and >95% ee.

![Scheme 14](image)

**Scheme 14.** Chemoenzymatic synthesis of (−)-Alloyohimbane and (−)-Yohimbane involving lipase-catalysed desymmetrisation of a diacetate intermediate.

The (1S,4R)-4-hydroxy-2-cyclopentenyl acetate is a valuable building block for the synthesis of prostaglandin analogues and other natural products such as cyclopentanoid derivatives (Hinze 2016). After screening different recombinant pig liver esterases (ECS-PLEs), one of this commercially available isoenzymes (ECS-PLE06) was selected to catalyse the hydrolytic desymmetrisation of cis-1,4-diacetoxy-2-cyclopentene at multigram scale, yielding the desired hydroxyester in high optical purity, that after crystallisation was obtained in enantiopure form (Scheme 15).

![Scheme 15](image)

**Scheme 15.** PLE-catalysed hydrolytic desymmetrisation of cis-1,4-diacetoxy-2-cyclopentene.
Pig liver esterase (PLE) has also hydrolysed selectively a prochiral diester precursor of BIRT-377 (Johnson 2015, Scheme 16), hydantoin that suppresses leukocyte adhesion serving as a potential candidate for the treatment of immune disorders and as an anti-inflammatory agent. In this case, the PLE used is a lyophilised mixture of isoenzymes obtained from a commercial source. After screening of cosolvents, temperatures and reaction times, the \((R)\)-carboxylic acid was isolated in 64\% yield and 70\% \(ee\) after 2 days of stirring at room temperature. Its optical purity was increased to >98\% \(ee\) after recrystallisation in a mixture of ethyl acetate and hexane, to perform later the total synthesis of the BIRT-377.

![Scheme 16. PLE-catalysed hydrolytic desymmetrisation of a BIRT-377 precursor.]

Telaprevir is a serine protease inhibitor currently approved for the treatment of hepatitis C (Scheme 17). Riva and co-workers have studied the synthesis of a key fragment in its stereoselective synthesis through desymmetrisation of a meso-diol using Amano PS as biocatalyst (Moni 2015). Employing vinyl acetate as both acyl donor and solvent, and the lipase supported on Celite, the \((1S,2R)\)-monoacetate was obtained in 97\% yield and 97\% \(ee\) after 17 h at 0 °C. In addition, the synthesis of the corresponding meso-diacetate was chemically performed to later study its hydrolytic desymmetrisation, leading in this case to the counterpart \((1R,2S)\)-monoacetate in 78\% yield and 95\% \(ee\) after 21 h at 20 °C.
Scheme 17. Enantioselective desymmetrisation of meso-compounds by acylation or hydrolysis for the synthesis of cis-2-[(hydroxymethyl)cyclopentyl]methyl acetate enantiomers.

2-Piperidones are valuable scaffolds present in many bioactive compounds such as cytisine, tacamonine or yaequinolone among others. Recently, the synthesis of (R)-1-benzyl-5-(hydroxymethyl)-2-piperidone has been achieved, finding the stereoselective acylation of N-benzyl-5-hydroxy-4-(hydroxymethyl)pentanamide as a key step in the synthesis (Khong 2016). The obtained (R)-monoacetate is an immediate precursor of the desired piperidone and, after testing CAL-B, PSL, Candida cylindracea (CCL) and AK lipase, the latest led to the best selectivities (Scheme 18). Then, an optimisation of the reaction conditions was performed, achieving the preparation of the (R)-monoacetate in 93% ee and 93% isolated yield after column chromatography when using 10 equivalents of vinyl acetate in acetonitrile. Alternatively, the (S)-monoacetate was also obtained through a complementary hydrolysis of the diacetate with the same lipase.

Scheme 18. Desymmetrisation of N-benzyl-5-hydroxy-4-(hydroxymethyl)pentanamide through lipase-catalysed acetylation.
Industrial researchers developed the kilogram synthesis of \((R)\)-allyl-[3-amino-2-(2-methylbenzyl)propyl]carbamate to support preclinical and clinical studies in an internal drug discovery program (Scheme 19). With that purpose, the alkoxy carbonylation of 2-(2-methylbenzyl)propane-1,3-diamine with allyl carbonate was studied, finding Amano PS as the best commercially available enzyme (Lindhagen 2016). Best conditions at preparative scale were found at 30 ºC and using 2-methyltetrahydrofuran (2-Me-THF) as environmentally friendly solvent, yielding the \((R)\)-carbamate in 82% ee, although its optical purity was improved to 88% ee after crystallisation as tartrate salt.

**Scheme 19.** PSL-catalysed desymmetrisation of 2-(2-methylbenzyl)propane-1,3-diamine.

The \((R)\)-isovaline has been reported to activate the metabotropic \(\gamma\)-aminobutyric acid-B receptor acting, for instance, as an analgesic agent (Scheme 20). Nojiri and co-workers have reported its chemoenzymatic synthesis in eight steps starting from diethyl 2-methylmalonate, where the key step is the introduction of chirality by desymmetrisation of 2-ethyl-2-methylmalonamide (Nojiri 2015). After screening 21 microorganisms and 2 amidases (CsAM from *Cupriavidus* sp. KNK-J915 and CnAM from *Cupriavidus necator* JMP134), the CsAM-catalysed hydrolysis of the prochiral diamide was successfully achieved on an 80 g-scale. After 22 h at 32 ºC, the \((S)\)-amido acid was obtained as crude product in full conversion and >98% ee, which was later transformed into the desired \((R)\)-isovaline through a chemical Hofmann rearrangement.
Scheme 20. Chemoenzymatic synthesis of (R)-isovaline involving the amidase-catalysed desymmetrisation of 2-ethyl-2-methylmalonamide.

3. Alcohol dehydrogenases

Alcohol dehydrogenases (ADHs), also known as carbonyl reductases or ketoreductases (KREDs), are a group of nicotinamide-dependent oxidoreductases that naturally catalyse the interconversion between alcohols and ketones or aldehydes, thus enabling the reduction of prochiral and racemic carbonyl compounds into optically active alcohols, or alternatively the selective oxidation of the hydroxyl group. Historically, ADHs have been the most demanding enzymes for the synthesis of chiral alcohols (Hollmann 2011), however the appearance of other enzymes such as esterases and lipases together with the discovery of their activities in organic media have made that both hydrolases and ADHs remain nowadays as first choices for the production of chiral alcohols.

Duloxetine is a blockbuster antidepressant drug that acts as a potent serotonin reuptake inhibitor and is employed in the treatment of several depression disorders. Its asymmetric synthesis has been extensively investigated in recent years (Larik 2016), representing the bioreduction of adequate precursors valuable examples of the potential of enzymes in asymmetric drug synthesis. A summary of biotransformations related to the synthesis of (S)-Duloxetine is reported in Table 3.
Table 3. Bioreduction of duloxetine precursors using ADHs.

![Bioreduction scheme](image)

<table>
<thead>
<tr>
<th>R</th>
<th>Enzyme</th>
<th>Conditions</th>
<th>$c$ (%)$^a$</th>
<th>$ee$ (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO$_2$Et</td>
<td>ChKRED15 S12G mutant</td>
<td>KPi buffer pH 7.0, 50 mM substrate Glucose, GDH 30°C, 6 h</td>
<td>&gt;99 (92)</td>
<td>&gt;99</td>
<td>Ren 2015</td>
</tr>
<tr>
<td>CO$_2$Me</td>
<td>ChKRED15 S12G mutant</td>
<td>KPi buffer pH 7.0, 50 mM substrate Glucose, GDH 30°C, 6 h</td>
<td>&gt;99 (94)</td>
<td>&gt;99</td>
<td>Ren 2015</td>
</tr>
<tr>
<td>CONHMe</td>
<td>ChKRED15 S12G mutant</td>
<td>KPi buffer pH 7.0, 250 mM substrate Glucose, GDH 30°C, 24 h</td>
<td>&gt;99 (93)</td>
<td>&gt;99</td>
<td>Ren 2015</td>
</tr>
<tr>
<td>CH$_2$NMe$_2$</td>
<td>E. coli/RtSCR9-GDH</td>
<td>KPi buffer pH 7.0, 1 M substrate Glucose 30°C, 8 h</td>
<td>&gt;99 (92)</td>
<td>&gt;99</td>
<td>Chen 2016</td>
</tr>
<tr>
<td>CN</td>
<td>Rhodotorula rubra MIM147</td>
<td>Tap water, 1% DMSO, 25 mM substrate Glucose 28°C, 24 h</td>
<td>&gt;99 (78)</td>
<td>99</td>
<td>Rimoldi 2016</td>
</tr>
</tbody>
</table>

$^a$Conversion values. Isolated yields in parentheses.

A variety of steroidal drugs have been synthesised using chemoenzymatic methods that involve the use of microorganisms for selective reductions. For instance, dehydroepiandrosterone (DHEA), also known as prasterone or 3β-hydroxyandrost-5-en-17-one, is an endogenous steroid hormone used as precursor of steroidal drugs for the treatment of different types of cancer (Scheme 21). Starting from 4-androstene-3,17-dione, a three-step synthesis has been reported for the synthesis of DHEA involving a basic isomerisation and two one-pot sequential steps: a regio- and stereoselective bioreduction followed by a chemical acetylation (Fryszkowska 2016). A colorimetric assay was developed to find good candidates...
in the bioreduction of the carbonyl located in the C-3 position, selecting *Sphingomonas wittichii* for an exhaustive optimisation in terms of substrate and enzyme loading, cosolvent, phase ratio, pH, reaction time and temperature. Thus, the enantiopure DHEA was obtained with full conversion after 21 h at 33 °C, which was isolated in 90% yield and 93% HPLC chemical purity.

![Scheme 21](image)

**Scheme 21.** Three-step synthesis of dehydroepiandrosterone acetate involving a bioreduction.

Romano and co-workers have reported the bioreduction of ethyl secodione, which gave access to a key intermediate in the synthesis of a wide panel of steroidal progestins used as hormonal contraceptives, such as desogestrel, gestodene, levonorgestrel or 3-keto-desogestrel (Scheme 22). The main complexity of this reaction is the possibility to obtain four different alcohol diastereoisomers, which were firstly chemically synthesised and characterised (Contente 2016). Initially, recombinant KRED1-Pglu led to the best results in terms of stereoselectivity, obtaining the desired (13R,17S)-alcohol with >98% *de* and >98% *ee* but only 65% conversion after 6 h. Unfortunately, the reaction did not proceed further at prolonged reaction times. Then, a screening of whole microbial cells was carried out finding also a complete stereoselectivity with *Saccharomyces cerevisiae* CEN.PK113-7D for a >95% conversion, while *Pichia minuta* CBS 1708 gave a similar conversion at a higher substrate concentration but with
lower enantioselectivity (92% ee).

**Scheme 22.** Bioreduction of ethyl secodione for steroid synthesis.

Also in this area, an engineered ketoreductase has been constructed together with a NADPH regeneration system into *Pichia pastoris*, where human 17β-hydrosteroid dehydrogenase type 3 and *Saccharomyces cerevisiae* glucose 6-phosphate dehydrogenase (G6PDH) were co-expressed (Shao 2016). This system has been applied in the efficient transformation of 4-androstene-3,17-dione in the hormone testosterone, an important pharmaceutical androgen steroid, avoiding the formation of by-products observed in the development of other bioreduction approaches, and obtaining testosterone with the highest productivity reported so far (2.33 g L\(^{-1}\) d\(^{-1}\), Scheme 23).

**Scheme 23.** Bioreduction of 4-androstene-3,17-dione into testosterone.

Sphingosine-1-phosphate (S1P) and its interaction with S1P receptors play a pivotal role in different biological processes such as cancer angiogenesis, heart development, lymphocyte horning and vascular
stabilisation. BMS-960 has been identified as a S1P receptor agonist and the (S)-4-(oxiran-2-yl)benzonitrile as a valuable intermediate in its synthesis (Scheme 24). This epoxide is ready available by a basic treatment of the 4-[(1S)-2-bromo-1-hydroxyethyl]benzonitrile, alcohol obtained thorough the bioreduction of the corresponding ketone (Hou 2017). After screening the activity of 250 ketoreductases (KREDs), 17 of them led to the enantiopure alcohol with complete conversion. For the multigram scale bioreduction reaction the NADH dependent KRED-110 was selected and, after the extraction of the desired alcohol, the formation of the epoxide was performed in the presence of sodium tert-butoxide to obtain more than 60 g of product in enantiopure form.

Scheme 24. Chemoenzymatic synthesis of a BMS-960 intermediate involving the bioreduction of 4-(bromoacetyl)benzonitrile and subsequent intramolecular cyclisation.

Alcohol dehydrogenases overexpressed in E. coli have efficiently catalysed the bioreduction of a panel of N-amino protected chloroketones for the formation of the corresponding halohydrins (Table 4), versatile intermediates in the synthesis of retroviral agents (de Miranda 2015). ADHs from Paracoccus pantotropous, Ralstonia species (RasADH), Sphingobium yanoikuyae (SyADH), Lactobacillus brevis (LBADH) and Rhodococcus ruber were tested against the ketones with different protecting groups, finding the best results with the Ralstonia species and Sphingobium yanoikuyae ADHs in 50 mg scale bioreductions towards both erythro and threo diastereomers (Table 4). A remarkable influence in the enzymatic activity was observed depending on the cosolvent employed for helping the substrate
solubilisation.

**Table 4.** Bioreduction of chloroketones bearing N-protecting groups in their structure.

<table>
<thead>
<tr>
<th>NHPG</th>
<th>Enzyme</th>
<th>Cosolvent</th>
<th>Conversion (%)</th>
<th>Alcohol de (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boc</td>
<td>RasADH</td>
<td>40% iPrOH</td>
<td>99 (82)</td>
<td>84 (R,S)</td>
</tr>
<tr>
<td>Boc</td>
<td>RasADH</td>
<td>50% EtOH</td>
<td>81 (70)</td>
<td>90 (R,S)</td>
</tr>
<tr>
<td>Cbz</td>
<td>RasADH</td>
<td>15% iPrOH + 20% DMSO</td>
<td>98 (80)</td>
<td>86 (S,S)</td>
</tr>
<tr>
<td>Moc</td>
<td>SyADH</td>
<td>5% iPrOH</td>
<td>97 (84)</td>
<td>90 (R,S)</td>
</tr>
</tbody>
</table>

Travoprost is a synthetic prostaglandin analogue used in the treatment of glaucoma and ocular hypertension (Scheme 25). Kroutil and co-workers developed its chemoenzymatic synthesis by means of the preparation of two chiral building blocks, which were obtained by redox processes (Holec 2015). On the one side, the meso-cyclopent-4-ene-1,3-diol was selectively oxidised using RasADH, obtaining the (R)-4-hydroxy-2-cyclopentanone in 82% isolated yield and 96% ee. On the other side, the bioreduction with LBADH of 1-[3-(trifluoromethyl)phenoxy]but-3-yn-2-one gave access to the enantiopure (R)-1-[3-(trifluoromethyl)phenoxy]butyn-2-ol.

**Scheme 25.** Enzymatic synthesis of two Travoprost fragments using alcohol dehydrogenases.
Our group recently reported a chemoenzymatic route towards the \((R)-3\)-methoxy-1-phenylethanol, which is an intermediate in the synthesis of \((S)\)-Rivastigmine, a drug employed in the treatment of dementia disorders. Thus, the deracemisation of 3-methoxy-1-phenylethanol gave access to the valuable \((R)\)-alcohol by a three-steps strategy (Scheme 26). This route involves the chemical oxidation of the alcohol to the \(3^{\prime}\)-methoxyacetophenone, destruction of the reactive iodide anions in the same pot and sequential bioreduction of the ketone (Méndez-Sánchez 2015). Therefore, the desired enantiopure alcohol was isolated in 99% yield after a liquid-liquid extraction.

**Scheme 26.** Chemoenzymatic deracemisation of 3-methoxy-1-phenylethanol, a Rivastigmine precursor.

4. Other classes of single enzymatic biotransformations.

In this section, the development of enzymatic methods for miscellaneous single transformations is reported. Therefore, the use of other less employed enzymes will be covered, which are microorganisms for selective hydroxylations, ammonia lyases towards the formation of \(C-N\) bonds, amino acid oxidases for the selective oxidation of \(C-N\) bonds, or amine transaminases for the transformations of ketones into chiral amines.

Hydroxylation of steroids is a challenging task for the discovery of new products with interesting pharmacological properties. For instance, microbial hydroxylation of epiandrosterone has been studied using *Aspergillus candidus* MRC 22634 obtaining 10 hydroxylated metabolites (Scheme 27). Three of
them were obtained as main products after selective monohydroxylation of the C-1, C-11 and C-15 position, in the latest compound occurring also the epimerisation of the C-3 (Yildirim 2017). The other seven products were obtained in 2-4% yield after a column chromatography separation of the reaction mixture.

![Scheme 27. Microbial hydroxylation of epiandrosterone with *Aspergillus candidus*.](image)

In another example, a peroxygenase produced by the ascomycetous fungus *Chaetomium globosum* has catalysed the hydroxylation of testosterone (Kiebist 2017, Scheme 28). Hydrogen peroxide was continuously supplied to the aqueous medium, obtaining with excellent diastereoselectivity the 4,5-epoxide of testosterone in β-configuration as the main product (61% isolated yield) and the 16α-hydroxytestosterone (7% isolated yield) with TTN of up to 7000 into the two oxygenated products.

![Scheme 28. Enzymatic hydroxylation of testosterone with a peroxygenase from *Chaetomium globosum*.](image)

Fasan and co-workers have improved previous low conversions attained in hydroxylation reactions by the use of cytochrome P450 monooxygenases variants as cells lyases (Tyagi 2016). Particularly, the
main focus was the scalable C-H hydroxylation of parthenolide, a lactone that is able to induce apoptosis in acute myeloid leukemia cells (Scheme 29). Therefore, different engineered enzyme preparations were tested as P450 lysates or as a two-plasmid system containing the P450 variant and the thermostable phosphite dehydrogenase (PTDH) as cofactor regeneration system expressed in *E. coli*, leading to the selective hydroxylation of parthenolide in the C-9 position. Once that 9-hydroxy-parthenolide was obtained, a wide panel of parthenolide analogues was synthesised via chemical acylation or O-H carbene insertion, and their antileukemic activities and toxicities against human umbilical cord blood cells were tested.

![Scheme 29](image)

**Scheme 29.** Enzymatic hydroxylation of parthenolide in the C-9 position and later chemical modification for the synthesis of antileukemic agents.

Ammonia lyases are enzymes that catalyse the asymmetric amination of unsaturated acids to yield optically active α-amino acids. Inside this family, methylaspartate ammonia lyase (MAL) is particularly attractive as provides an efficient tool for the synthesis of nitrogenated compounds with excellent selectivities. Poelarends and co-workers have investigated the chemoenzymatic synthesis of ortho-, meta- and para-monosubstituted L-threo-3-benzyloxyaspartate derivatives, which possess potential applications as glutamate transporter blockers (de Villiers 2015). Two genetically modified MALs (L384A and L384G) were employed for this stereoselective transformation using a great excess of
ammonia as amine donor (Scheme 30). Eight out of ten substrates tested, bearing F, CF\textsubscript{3} and CH\textsubscript{3} substituents at the aromatic ring, gave conversions in the range 91-95% and complete diastereo- and enantioselectivity (>95% de, >99% ee).

\textbf{Scheme 30.} Synthesis of L-threo-3-benzzyloxyaspartate derivatives using MAL mutants.

Two complementary biocatalytic strategies are described for the synthesis of both enantiomers of 4-bromophenylalanine by using phenylalanine ammonia lyases (PALs) or D-amino acid dehydrogenases (DAADHs, Ahmed 2015). On one hand, the asymmetric hydroamination of 4-bromocinnamic acid was studied with six PALs at 5 mM substrate concentration, obtaining the best conversion (80%) towards the L-amino acid with the PAL from the cyanobacterium \textit{Anabaena variabilis} with a mutation in F107A (\textit{AvPAL}-F107A). On the other hand, the reductive amination of 4-bromophenylpyruvic acid led to the D-amino acid with complete conversion and total selectivity using an engineered DAADH from \textit{Corynebacterium glutamicum}. Interestingly, the L-isomer was used as an intermediate in the synthesis of a dipeptidyl peptidase 4 (DPP IV) inhibitor (Scheme 31).

\textbf{Scheme 31.} Use of phenylalanine ammonia lyases and amino acid dehydrogenases for the synthesis of 4-bromophenylalanine enantiomers.
AvPAL has also served as efficient biocatalyst for the synthesis of 12 enantiopure ring-substituted L-pyridylalanines and 5 different L-heteroarylalanines through a telescopic strategy that involves a chemical Knoevenagel-Doebner condensation followed by a biocatalytic hydroamination (Scheme 32). In general, excellent conversions and enantioselectivities up to >99% were achieved, although the products were recovered in 32-60% isolated yield after column chromatography (Ahmed 2016). The substrates that displayed a worse enantioselectivity were submitted to an additional step of deracemisation cascade employing an aminoacid acid oxidase (DAAO) coupled with ammonia-borane, thus obtaining the desired products with >99% ee in every case.

**Scheme 32.** Chemoenzymatic cascade synthesis of L-heteroarylalanines through Knoevenagel-Doebner chemical condensation and PAL-catalysed hydroamination.

Transaminases (TAs) are versatile biocatalysts able to transform prochiral ketones into enantiomerically pure amines in theoretically 100% yield. Since the last decade, this type of enzymes has received considerable attention, finding wide application in the synthesis of chiral drugs (Fuchs 2015). For instance, Sitagliptin is another DPP-4 inhibitor used in the treatment of diabetes an oral anti-diabetic drug, marketed as Januvia (Scheme 33), which is a blockbuster in pharma industry. After an exhaustive study of the biotransamination of 11 ketoesters for the formation of the corresponding optically active amino esters using the transaminase ATA117-rd11, the best conditions were found for the hydroxyethyl-3-oxo-4-(2,4,5-trifluorophenyl)butanoate (Hou 2016). The (R)-amino ester was obtained in 99% ee and 82% conversion after 24 h, using DMSO as cosolvent to assure a good solubility at 100 mM substrate concentration in the reaction with isopropylamine (1 M) as amine donor.
Scheme 33. Biotransamination of hydroxyethyl-3-oxo-4-(2,4,5-trifluorophenyl)butanoate for the stereoselective synthesis of a Sitagliptin precursor.

The 8-azabicyclo[3.2.1]octane core constitutes a structural motif within different neuroactive compounds such as cocaine and atropine (Scheme 34). Protein engineering combining rational design with directed evolution has been performed over the (S)-selective TA from Ruegeria sp. TM1040 in order to find an efficient catalyst for the transformation of 8-benzoyl-8-azabicyclo[3.2.1]octan-3-one into the 3-amino-8-azabicyclo[3.2.1]oct-8-yl-phenyl-methanone. Then, it was possible to obtain a TA variant with five mutated amino acids (Y59W/Y87F/Y152F/T231A/I234M) that allows complete diastereoselectivity towards the formation of the exo-amine (>99% de, Weiß 2016).

Scheme 34. Biotransamination of 8-benzoyl-8-azabicyclo[3.2.1]octan-3-one with different TA variants from Ruegeria sp. TM1040.

(−)-Pinidinone is a defensive alkaloid with interesting biological properties, and its chemoenzymatic
synthesis has been recently described through a transaminase triggered aza-Michael strategy starting from a dimethyl ketoenone (Scheme 35). The reaction was efficiently catalysed by the commercially available ATA-117 in the presence of just 2 equivalents of isopropylamine as amine donor, which allow the selective modification of the methyl ketone leading to the \((R)\)-amine intermediate (Ryan 2016). Following the biotransamination, a spontaneous intramolecular aza-Michael reaction (IMAMR) occurred providing a mixture of the optically active \(cis\) and \(trans\)-amino ketones that were converted into the desired \(cis\)-(\(R,R\))-isomer by epimerisation of the \(trans\)-isomer in methanol.

**Scheme 35.** Biotransamination/IMAMR cascade for the synthesis of \((-\))-Pinidinone.

5. Multienzymatic systems for the development of cascade and sequential processes.

Nowadays, there is a clear trend in the design of concurrent processes in order to carry out multiple transformations without the requirement of reaction intermediate isolations, which are time-consuming and yield-reducing steps in synthetic routes. In addition, unstable compounds can be considered in the reaction sequence, making possible the design of more straightforward routes. In this section, the use of two or more biocatalyst has been considered for challenging stereoselective transformations.

Profen derivatives have attracted great attention due to their anti-inflammatory properties. A *one-pot* methodology has been disclosed for the deracemisation of \((\pm)\)-2-phenyl-1-propanol by combining the use of *Trametes versicolor* laccase (TvL) and a selective ADH (Díaz-Rodríguez 2015). The system formed by TvL and the chemical mediator TEMPO is responsible of the non selective oxidation of the alcohol into the 2-phenylpropanal (Scheme 36), while the proper selection of the reductive enzyme led
to the formation of the (S)- or the (R)-alcohol in high conversions (84-85%) and good selectivities (82-86% ee) through a DKR protocol. This one-pot methodology was successfully applied in 150 mg substrate scale to obtain both enantiomers with an adjustment of the pH from acidic to basic after the oxidation step (3.5 h). Then, the ADH was added and the corresponding optically active alcohol was obtained in 72% yield with evo-1.1.200 and in 71% yield with horse liver ADH (HL ADH).

**Scheme 36.** Deracemisation of the profenol core using a laccase and an ADH.

The microbial hydroxylation of DHEA has been studied by incubation with *Beauveria bassiana* ATCC 7159 finding two main products that were separated by column chromatography (Scheme 37). These are androstenediol, obtained by reduction of the ketone at the C-17 position, and 3β,11α,17β-trihydroxyandrost-5-ene, through an additional C-11 selective hydroxylation, which were isolated in moderate yields after 7-days incubation at 26 °C and pH 7 (Gonzalez 2017).

**Scheme 37.** Microbial hydroxylation of DHEA with *Beauveria bassiana*.

Bimatoprost and Latanoprost are prostaglandin analogues used for the treatment of ocular hypertension and glaucoma (Scheme 38). After an exhaustive screening of microorganisms, Romano and co-workers have described the used whole cells of *Pichia anomala* yeast for the production of Lactonodiol B and
Lactonodiol L, which are precursors of Bimatoprost and Latanoprost, respectively (Contente 2015). In these biotransformations, the ratio of products depended on the relative enoate and carbonyl reductase activities, which were modulated by the addition of different co-substrates for co-factor regeneration. In addition, the whole cells displayed esterase activity and catalysed the hydrolysis of the benzoate-protecting group. The two-step hydrolysis-bioreduction led to Lactonodiol B in 62% yield and 97% de by adding glycerol as co-substrate, while the use of fumaric acid gave 82% yield of Lactonodiol L with 97% de in a three-step biotransformation.

![Scheme 38](image)

**Scheme 38.** Production of Lactonodiol B (with glycerol as co-substrate, top) or Lactonodiol L (with fumaric acid, bottom) using *P. anomala* whole cells.

Co-expression of various enzymes in a single plasmid allows the development of enzymatic cascades in shorter reaction times and enhanced productivity. Kroutil and co-workers have also taken advantage of this methodology by preparing a three-enzyme catalyst in *E. coli* cells (Gourinchas 2015). Using a two-step redox cascade, L-amino acids were converted into optically pure (S)- and (R)-hydroxy acids, that are valuable building blocks in medicinal chemistry. Different expression constructs were designed in order to obtain good conversion values, which is depending on the gene position in the plasmid. The system was formed by (i) an L-AAD from *Proteus myxofaciens* to convert the amino acid in ketoacid;
(ii) a L-Hic from *Lactobacillus confusus* DSM 20196 or D-Hic from *Lactobacillus paracasei* DSM 20008 for the selective reduction of the ketoacid; (iii) and a FDH from *Candida boidinii* for the regeneration of the NADH cofactor. Using 100 or 200 mM substrate concentration, three L-amino acids were completely converted into the corresponding optically active hydroxy acids (98-99% ee), which were isolated in 71-86% without requiring chromatographic purification. The synthesis of (S)-p-hydroxyphenyl lactic acid was achieved using this catalytic system, a precursor of pharmaceuticals such as the antidiabetic saroglitazar (Scheme 39).

Scheme 39. Three-enzyme biocascade for the conversion of L-tyrosine into enantiopure (S)-p-hydroxyphenyl lactic acid.

The same research group has described the synthesis of enantiopure p-hydroxyphenyl lactic acid and aryl-substituted derivatives by an enzymatic cascade reaction involving three steps: (i) C-C coupling of phenol derivatives with pyruvate in the presence of ammonia; (ii) oxidative deamination; and (iii) stereoselective reduction (Scheme 40). These three reaction steps are consecutively catalysed by a tyrosine phenol lyase (TPL) mutant M379V from *Citrobacter freundii*, L-amino acid deaminase (L-AAD) from *Proteus myxofaciens* and stereocomplementary L- or D-isocaproate reductases (Hic) from *Lactobacillus confusus* DSM 20196 or D-Hic from *Lactobacillus paracasei* DSM 20008, while the cofactor recycling was performed with a commercially available formate dehydrogenase (FDH). The
reactants were the $p$-unsubstituted phenol substrate and just pyruvate, molecular oxygen and ammonium formate, yielding both antipodes of seven L-hydroxy acids in 96->97% ee and 58-85% isolated yield (Busto 2016).

Scheme 40. Transformation of phenols into optically active $p$-hydroxyphenyl lactic acids using a three-step enzymatic sequence.

Both cis-enantiomers of osmundalactone, a hydroxypyranone natural product also present in the structure of angiopterlactones, were synthetised by a chemoenzymatic strategy involving a biocascade (Blume 2016, Scheme 41). The transformation of 2-acetylfuran into 6-hydroxy-2-methyl-2H-pyran-3(6)-one is based in a two-step three-enzyme cascade consisting in (i) asymmetric reduction of the acetyl group using a commercially available ADH; (ii) action of a chloroperoxidase from C. fumago and a glucose oxidase from A. niger for the Achmatowicz-type ring expansion. A final iridium-based redox dynamic stereoconvergent isomerisation affords the desired cis lactones with excellent selectivity. The enantiocontrol of the reaction is defined by the use of the ADH, ($R$)-alcohol with evo-1.1.200 (99% ee) or ($S$)-alcohol with evo-1.1.030 (99% ee), which led to the 4-epi(-)-osmundalactone or the 4-epi-(+)-osmundalactone, respectively.
Scheme 41. Biocascade followed by iridium-based redox isomerisation for the synthesis of (−)-cis-osmundalactone.

Finally, the combination of a transaminase and a strictosidine synthase will be discussed for the preparation of C-3 methylated strictosidine derivatives through a cascade approach (Fischereider 2016). (S)-Strictosidine is a pivotal building block for the synthesis of many indole alkaloids with remarkable properties in the treatment of different illness (Scheme 42). The stereoselective synthesis of optically active C-3 methylated derivatives has been possible through a two-step cascade involving the biotransamination of prochiral ketones for the formation of the enantiopure (R)- or (S)-amines depending on the enzyme selectivity, which were later converted into diastereomerically pure products through a Pictet-Spengler reaction catalysed by strictosidine synthase.

Scheme 42. Two-step enzymatic cascade for the synthesis of C-3 methylated strictosidine derivatives.
6. Proper selection of the enzymatic step and the class of biocatalyst

The identification of a viable retrosynthetic analysis is a key issue in synthetic chemistry. Enzymes provide a plethora of solutions, the scientist trying to identify the best synthetic routes regarding selectivity, isolated yield or sustainability among other parameters. As mentioned in previous sections, hydrolases has been extensively reported as excellent candidates for KRs of racemates, although their application is usually hampered for the theoretically maximum 50% isolated yield of classical KRs. Alcohols dehydrogenases and transaminases represent excellent alternatives for alcohol and amine syntheses as the transformation of prochiral compounds into enantiopure valuable products is possible in 100% conversion. This section is focused on the study of complementary approaches for the stereoselective production of drug precursors, and the results will be analysed in terms of selectivity and productivity values.

Imidazole containing drugs such as miconazole, econazole or sertraconazole are effective antifungal agents (Scheme 43), which in most of the cases are commercialised as racemates for the treatment of vaginal and skin fungal infections. Halohydrins have been identified as adequate precursors for the synthesis of these azole derivatives, the use of biocatalytic methodologies representing an elegant alternative for asymmetric synthetic purposes (Mangas-Sánchez, 2012). In this context, the use of lipases and alcohol dehydrogenases has been recently evaluated in the production of optically active 2-chloro-(2,4-chlorophenyl)ethanol. On the one hand, the use of an immobilised hydrolase such as the PFL failed in the resolution of the racemate via acetylation with vinyl acetate, while better results were attained with less hindered substrates (Ferreira 2017). On the other hand, the bioreduction of the corresponding ketone with lyophilised E.coli cells expressing a ketoreductase from Scheffersomyces stiptis CBS 6045 gave enantiopure (R)-2-chloro-(2,4-chlorophenyl)ethanol in 88% isolated yield. A STY up to 268 g L\(^{-1}\) d\(^{-1}\) was achieved without requirement of external cofactor addition, reaching an excellent E factor of 7.25 when excluding the role of water (Shang 2017).
Scheme 43. Bioreduction of 2-chloro-1-(2,4-chlorophenyl)ethanone for the synthesis of a chiral intermediate of azole drugs.

Levofloxacin is a potent fluoroquinolone antibacterial agent employed in the treatment of pneumonia, sinusitis or urinary tract infections among others (Scheme 44 top). Our research group have recently described the successful use of *Rhizomucor miehei* lipase (RML) under hydrolytic conditions and the commercially available alcohol dehydrogenase evo-1.1.200 in bioreduction experiments for the asymmetric synthesis of \((R)-1-(2,3\text{-difluoro}-6\text{-nitrophenoxy})\text{propan-2-ol} \) (López-Iglesias 2015). In the first case, RML catalysed the hydrolysis of a racemic acetate leading to the formation of the enantiopure \((R)\)-alcohol in 45% yield. The development of a bioreduction process, instead of a classical KR, allowed the formation of the desired \((R)\)-alcohol in a higher yield (94%) after 24 h at 30 °C.

Alternatively, we have also investigated the potential of lipases and transaminases for the production of a chiral amine Levofloxacin precursor in order to reduce the number of steps for the synthesis of this drug (Mourelle-Insua 2016). While the lipase-catalysed resolution of racemic 1-(6-bromo-2,3-difluorophenoxy)propan-2-amine resulted inefficient due to the unstability of the amine in the reaction medium, the biotransamination of 1-(6-bromo-2,3-difluorophenoxy)propan-2-one led to the desired enantiopure \((S)\)-amine in 61% isolated yield by using commercially available ATA-256 as biocatalyst and isopropylamine as amine donor (Scheme 44 bottom).
Ticagrelor is a platelet aggregation inhibitor approved for the treatment of acute coronary syndrome. A retrosynthetic analysis revealed that the cyclopropyl subunit is a good starting for the introduction of chirality by means of different strategies (Scheme 45 top), such as ketone reduction, amide hydrolysis or ester hydrolysis (Hugentobler 2016). Firstly, the bioreduction of 1-(3,4-difluorophenyl)-3-nitropropan-1-one occurred with excellent conversion and selectivity towards the corresponding (S)-alcohol, while poorer values were obtained when preparing its counterpart, but an extremely high loading of ADH was required. Secondly, the microorganism *Rhodococcus rhodocrous* acted with either low selectivity or activity in the hydrolysis of the racemic amide intermediate. Finally, *Thermomyces lanuginosus* lipase (TLL) was identified a good candidate for the resolution of the ester precursor through a hydrolytic procedure (Scheme 46 middle), although the (1S,2S)-acid was obtained in low conversion. Significantly, the same authors reported the comparison between a batch reactor process with an immobilised TLL
and a flow chemistry approach, obtaining a significant reduction of the reaction time and higher conversions in the flow reaction (Hugentobler 2017). In both cases, the reusability of the lipase was successfully achieved and gave access to the (1R,2R)-ester that is easily transformed to a targeted (1R,2S)-amine, key Tricagelor precursor.

More recently, Arnold and co-workers have reported the use of an engineered hemoglobin of *Bacillus subtilis* for the cyclopropanation of 3,4-difluorostyrene with ethyl diazoacetate on a preparative scale (Hernandez 2016, Scheme 45 bottom). Thus, after performing site-directed mutagenesis and exploring the potential of different mutants, ethyl (1R,2R)-2-(3,4-difluorophenyl)-cyclopropane carboxylate was obtained in 79% yield and with excellent diastereo- (>99% dr) and enantioselectivity (98% ee) when using the Y25L, T45A and Q49A triple mutant.

![Ticagrelor](image)

**Scheme 45.** Biocatalytic methods for the synthesis of Tricagelor intermediates by using an alcohol
dehydrogenase (top), a lipase in a flow setup (middle) or a truncated globin (bottom).

Profen drugs are currently widely employed for their anti-inflammatory applications, the flurbiprofen being one of the most common representatives of this drug family. Its therapeutic action resides mainly in the S-enantiomer while the R-enantiomer has adverse gastrointestinal effects. Recently, the behaviour of the Candida species lipase (CSL) in the presence of a set of ionic liquids has been studied (Scheme 46 top). Moderate selectivities were observed in the esterification of flurbiprofen using 10 equivalents of methanol (E<23), yielding with the [bmim][PF₆] the desired (S)-carboxylic acid in enantiopure form although in low yield (Zhao 2017).

Scheme 46. Enzymatic synthesis of flurbiprofen enantiomers by lipase-catalysed esterification of racemic flurbiprofen in ionic liquids (top) or decarboxylation of the flurbiprofen malonate (bottom).

In this context, several profen drugs have been investigated by resolution of ethyl ester racemates, finding a great improvement when engineering new mutants of the Yarrowia lipolytica lipase were employed rather than the wild-type enzyme (Gérard 2017). The enantioselectivities of the hydrolytic reactions over ibuprofen, naproxen and ketoprofen ethyl esters were highly enhanced after site-directed mutagenesis and bioinformatics analyses. More recently, Kourist and workers described the use of the bacterial arylmalonate decarboxylase (AMDase) from Bordetella bronchiseptica for the enantioselective decarboxylation of prochiral arylmalonates, leading to both enantiomers of flurbiprofen in very short
reaction times (Gaßmeyer 2016). The wild type selectively catalysed the formation of the (R)-flurbiprofen, so this was a good starting point for site-directed mutagenesis experiments achieving an improvement of the activity but also changing the selectivity for the production of the (S)-enantiomer (Scheme 46 bottom). The best results were found with the (S)-selective AMDase variant G74C, M159L, C188G, V43I, A125P and V156L and the (R)-selective AMDase variant V43I, A125P, V156L and M159L that led to both flurbiprofen enantiomers in 98% ee and 95% and 99% yield, respectively.

Ivabradine is employed in the treatment of myocardial ischemia especially when it is not fully managed by β-blockers as it reduces the heart rate without loss of cardiac contractility (Scheme 47). Recently, three independent enzymatic approaches were employed in the synthesis of appropriate chiral intermediates by means of lipase-catalysed resolution, bioreductions or biotransamination experiments (Pedragosa-Moreau 2017). An exhaustive screening was performed for the resolution of a racemic amine intermediate by means of acylation and alkoxycarbonylation processes using a set of lipases, solvents and chemical reactants. The best results were obtained with a variety of Pseudomonas cepacia lipases, 2-methyl-tetrahydrofuran (2-Me-THF) as solvent and ethyl carbonate as resolving agent, yielding the unreacted (R)-amine and the desired (S)-carbamate in moderate to good enantiomeric excess values.
Enzymatic approaches for the synthesis of Ivabradine precursors by means of lipase and transaminase-catalysed processes.

Scheme 47. Enzymatic approaches for the synthesis of Ivabradine precursors by means of lipase and transaminase-catalysed processes.

Alternative enzymatic strategies were investigated, selecting in this case the corresponding aldehyde as starting material. Unfortunately, none of the selected ADHs gave access to the alcohol with any selectivity, while in the biotransamination DKR-process with transaminases (TAs) excellent conversion and enantiomeric excess values up to >99 and 78% were achieved for the (S)- and (R)-amine, respectively. This synthetic approach was scaled-up to obtain the desired enantiomer with 90% ee that was converted into the enantiopure Ivabradine hydrochloride in a four-step sequence, and an intermediate recrystallisation, without the need of chromatography purification.

Rasagiline is a potent inhibitor of the monoamine oxidase type B used in the treatment of Alzheimer’s disease (Scheme 48), the (R)-configuration of the stereogenic center is vital for its inhibitory activity. Sousa and co-workers have reported two complementary methods to access an alcohol derivative by means of Candida antarctica lipase type B (CAL-B)-catalysed transesterification or hydrolysis of the
corresponding racemic alcohol and acetate, respectively (Sousa 2015). In both cases, conversions around 49% were attained and the products where obtained in optically pure form while the remaining substrates were recovered with 94% ee. Interestingly, the immobilised Thermomyces lanuginosus lipase (TLL) has been found as a selective biocatalyst for both the transesterification and the hydrolytic reaction (Fonseca, 2015). Therefore, in the transesterification reaction with 5 equiv. of vinyl acetate and hexane as solvent, just 15 minutes at 35 ºC were required for a 50% conversion isolating the (S)-alcohol and the (R)-acetate in enantiopure form (Scheme 48 top). Similarly, the hydrolysis of the racemic acetate led to the (R)-alcohol and the (S)-acetate in 96 and 93% ee, respectively after 24 h at 30 ºC in a mixture of phosphate buffer and THF (80/20, v/v). More recently, an alternative approach for the stereoselective synthesis of Rasagiline and its enantiomer has been reported (Matzel 2017). The reductive amination between 1-indanone and propargylamine has been successfully conducted using an imine reductase from Nocardi cyriacigeorgica GUH-2 (IREDC-14), obtaining the Rasagiline in 56% isolated yield (71% conversion) and 90% ee after precipitation as hydrochloride salt (Scheme 48 bottom). Alternatively, the (S)-enantiomer was prepared in 81% yield (91% conversion) and 72% ee using the IRED-Sip from Streptomyces ipomoeae 91-03.

Scheme 48. Stereoselective synthesis of Rasagiline by reductive amination using an imine reductase, and lipase-catalysed resolution of an alcohol precursor using CAL-B.
Nowadays, statins are a highly demanding class of lipid-lowering medication used for the treatment of high cholesterol levels in blood and prevent cardiovascular diseases. Atorvastatin and Rosuvastatin are blockbuster drugs whose structures possess a common linear chain that can be obtained from the ethyl (S)-4-chloro-3-hydroxybutyrate. This intermediate has been traditionally obtained through the bioreduction of ethyl 4-chloro-3-oxo-butanoate (COBE) using different ADHs (Table 5).

**Table 5. Bioreduction of Atorvastatin precursors using ADHs.**

<table>
<thead>
<tr>
<th>[Ketoester] (M)</th>
<th>Enzyme</th>
<th>Conditions</th>
<th>Alcohol yield (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Alcohol ee (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>Surf-CRS-GDH</td>
<td>KPi buffer pH 6.5</td>
<td>&gt;99 (96)</td>
<td>&gt;99</td>
<td>Srivastava 2015</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bu&lt;sub&gt;2&lt;/sub&gt;O NADP&lt;sup&gt;+&lt;/sup&gt;, Glucose 30 ºC, 10.5 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>RpCR-GDH</td>
<td>KPi buffer pH 7.0 Toluene</td>
<td>&gt;99 (91)</td>
<td>&gt;99</td>
<td>Xu 2016</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Substrate feeding Glucose 30 ºC, 180 rpm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td><em>Lactobacillus curiae</em> S1L19</td>
<td>KPi buffer pH 7.0 NAD&lt;sup&gt;+&lt;/sup&gt; Glucose 30 ºC, 4 h</td>
<td>&gt;99</td>
<td>99</td>
<td>Zhang 2016</td>
</tr>
</tbody>
</table>

<sup>a</sup> Isolated yields in parentheses.

Alternatively, the versatility of the corresponding δ-ketal-β-keto ester has been demonstrated as its bioreduction was developed in a completely stereoselective manner and with full conversion (Tartaggia 2016). After screening a commercial kit of reductases, the KRED-130 gave access to the enantiopure (R)-δ-ketal-β-hydroxy ester in 86% isolated yield in a 32 mmol scale (Scheme 49).
Scheme 49. Bioreduction of a δ-ketal-β-keto ester for the synthesis of statins.

(S)-4-Chloro-3-hydroxybutyronitrile is an alternative building block of Atorvastatin, through the immediate precursor ethyl (R)-4-cyano-3-hydroxybutyrate, and its traditional synthesis involves the resolution of the epichlorohydrin using metal complexes or enzymes, to later develop a cyanation reaction. Unfortunately, this is limited for the maximum 50% yield inherent to KRs. The production of (S)-4-chloro-3-hydroxybutyronitrile using halohydrin dehalogenases (HHDH) has been reported through a one-pot two-steps sequence, which involves an enzymatic dehalogenation and later chemical cyanation (Wan 2015). An exhaustive study in terms of HHDH screening and evolution, plus further optimisation of reaction parameters such as pH, reaction time and NaCN content (Scheme 50). The use of the halohydrin dehalogenase HheC from Agrobacterium radiobacter AD1 with the mutation W249F led to the desired nitrile in 86% isolated yield with 97.5% ee in a 4 g scale.

Scheme 50. Synthesis of an Atorvastatin precursor using HHDH.

7. Conclusions

The versatility of enzymes for stereoselective transformations has been reviewed, trying to provide an actual scenario of the potential of these types of catalysts for the synthesis of pharmaceuticals and valuable natural products. Single enzymatic transformations have been firstly considered for the
asymmetrisation of intermediate and final drugs in a straightforward manner. The role of lipases and ADHs is crucial for the selective production of structurally different families of organic compounds, while also other classes of enzymes such as transaminases, lyases and diverse types of oxidoreductases are attracting great attention in recent years.

The aim of this review was not only to provide a collection of successful transformations, but also identify practical limitations and discuss the advantages of complementary enzymatic methods. In this context, some aspects of the biocatalytic methods must be enhanced to improve the sustainability of the processes, such as the reduction of large excess of reactants or cosubstrates and the development of efficient cofactor recycling systems, the shortening of reaction times without the use of volatile and toxic organic solvents or the avoidance of chromatographic separations for product purification. Without any doubt, the use of novel reagents and catalysts, application of neoteric solvents or the generation of evolved enzymes is nowadays paving the way for biotransformation improvements. For that reason, special efforts have been made in the last part of the review to describe the design of concurrent processes, which made possible complex transformations by making compatible the action of various catalysts through cascade or sequential processes. This provides additional advantages to chemical routes as the isolation of intermediates, which sometimes are not stable, is not necessary improving essential aspects in synthetic chemistry such as time, economy and wastes.

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Declaration of interest

The authors report no declarations of interest.
References


Dwivedee BP, Ghosh S, Bhaumik J, Banoth L, Banerjee UC. 2015. Lipase-catalyzed green synthesis of
enantiopure atenolol. RSC Adv 5: 15850-15860.


